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(54) Title: A PHARMACEUTICAL COMPOSITION CONTAINING EZRIN MUTATED ON TYROSINE 353

(57) Abstract

The invention relates to a pharmaceutical composition containing an effective amount of ezrin mutated on tyrosin 353, or a functional fragment or derivative thereof, in association with a pharmaceutically acceptable carrier, for use preferably in the prevention and/or treatment of tumors.

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- 1 -

A pharmaceutical composition containing ezrin mutated on tyrosine 353"

The present invention relates to a pharmaceutical composition containing ezrin mutated on tyrosine 353, for controlling cell survival and apoptosis and for use preferably in the prevention and/or treatment of tumors.

5 Ezrin was characterized as a component of brush-border and placental microvilli in the early 1980s (Bretscher et al., 1983). The family now consists of four members in vertebrates, namely ezrin, radixin, moesin (ERM proteins) and merlin (moesin-ezrin-radixin-like protein : also named schwannomin).

10 The structure of all family members consists of an amino-terminal globular domain followed by an α -helical region and a carboxy-terminal domain. The family belongs to the band 4.1 superfamily on the basis of sequence homology of the amino-terminal domain with the erythrocyte membrane-cytoskeleton linker protein band 4.1

15 Ezrin, radixin and moesin are thought to work as cross-linkers between plasma membranes and actin-based cytoskeletons (Arpin et al., 1994)..

Phosphorylation may regulate the activity of the proteins triggered by several growth factors. ERM proteins could be regulated by phosphorylation of different protein domains, including Tyr145 located in the amino-terminal domain and Tyr353 in the α -helical domain. These two residues are phosphorylated by EGF receptor (Krieg et al. (1992)). Phosphorylation of a threonine residue in the carboxy-terminal domain may also regulate the actin-binding ability of ERM proteins (Vaheri et al., 1997, Tsukita et al., 1997).

25 The authors of the present invention have recently shown that ezrin is an effector of hepatocyte growth factor-mediated migration and morphogenesis in epithelial cells (Crepaldi et al., 1997).

30 The authors of the present invention have now discovered that ezrin mutated on tyrosine 353 as shown on figure 1 impairs the ability of cells to survive in a collagen matrix, more particularly in a collagen type I matrix, and induces apoptosis, that is to say cell death. Such apoptosis could be due to a

disruption of the signal transmission mediated by molecules of adhesion. *In vivo*, normal cells lie on a basal membrane which does not contain collagen type I. Tumor cells, and more particularly metastatic cells, may contact the extracellular matrix which contains collagen type I and are thus sensitive to the effect of ezrin mutated on tyrosine 353. Consequently ezrin mutated on tyrosine 353 is a good candidate for the prevention and/or treatment of tumors. Furthermore, ezrin mutated on tyrosine 353 could be useful to prevent metastasis and/or to lead to the apoptosis of migrating tumor cells involved in metastasis.

A subject of the present invention is thus a pharmaceutical composition containing an effective amount of ezrin mutated on tyrosine 353, or a functional fragment or derivative thereof, in association with a pharmaceutically acceptable carrier.

A further subject of the present invention is a pharmaceutical composition containing an effective amount of DNA or RNA encoding ezrin mutated on tyrosine 353, or encoding functional fragments or derivatives thereof, in association with a pharmaceutically acceptable carrier.

The pharmaceutical compositions of the invention are more particularly useful for the prevention and/or treatment of tumors, namely for the prevention and/or treatment of metastasis.

The aminoacid sequence of native human ezrin is shown on figure 1. The expression "ezrin mutated on tyrosine 353" or "Y353 ezrin mutant" refers to a polypeptide having the sequence shown of figure 1, except for the aminoacid 353 which is different from a tyrosine residue.

The aminoacid 353 of the Y353 ezrin mutant may be preferably a phenylalanine residue, but may also be any other aminoacid residue which cannot be phosphorylated.

Said Y353 ezrin mutant may be produced from native ezrin by site-directed mutagenesis, which is a technique well-known in the art (Crepaldi et al., 1997).

The term "functional derivative" is understood to refer to any polypeptide variant of Y353 mutant or any molecule resulting from a genetic and/or chemical modification of Y353 ezrin mutant, that is to say obtained by mutation, deletion, addition, substitution and/or chemical modification of a single or of a limited number of aminoacids, as well as any isoform sequence, that is to say a sequence which is identical to the Y353 ezrin mutant sequence, except for one or more aminoacids in the form of the D enantiomer, the said isoform, modified or variant sequences having conserved the biological activity of the Y353 ezrin mutant.

The biological activity of the Y353 ezrin mutant refers to the ability of Y353 ezrin mutant to induce apoptosis.

More particularly the biological activity of the Y353 ezrin mutant refers to the property of the Y353 ezrin mutant to impair the ability of cells to survive in a collagen matrix, more particularly in a matrix containing collagen type I or to grow in aggregates in suspension. This indicates that signalling from cell-cell and cell-substratum contacts is impaired.

The term "derivatives" thus comprises any polypeptide having an aminoacid sequence which is substantially identical to the Y353 ezrin mutant in which one or more residues have been conservatively replaced by a functionally similar residue and which demonstrates its ability to mimic the Y353 ezrin mutant as described in the present invention. Examples of conservative replacements include the replacement of a hydrophobic residue such as isoleucine, valine, leucine or methionine with another hydrophobic residue, the replacement of a polar residue such as arginine with lysine, glutamine with asparagine or glycine with serine, the replacement of a basic residue such as lysine, arginine or histidine with another basic residue or the replacement of an acidic residue such as aspartic acid and glutamic acid with another acidic residue.

Similarly, the term "derivatives" comprises any polypeptide having one or more residues which are derived chemically from Y353 ezrin mutant by reaction of a functional group. Such derived molecules include, for example,

molecules in which the free amino groups have been substituted in order to form amine hydrochlorides. The free carboxylic acid groups may be derived in order to form salts, methyl or ethyl esters or other types of esters or hydrazides. The free hydroxyl groups may be substituted in order to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of the histidine may be substituted in order to form N-imidazole benzylhistidine. Peptides which contain one or more derivatives of an amino acid in its natural form from the 20 natural amino acids are also included as chemical derivatives. For example, proline may be replaced with 4-hydroxyproline, lysine may be replaced with 5-hydroxylysine ; histidine may be replaced with 3-methyl-histidine ; serine may be replaced with homoserine ; and lysine may be replaced with ornithine.

The term "functional fragment" is understood to refer to any polypeptide having an aminoacid sequence selected from any part of Y353 ezrin mutant sequence, and containing of course said aminoacid 353. Preferred fragments contain at least the aminoacid sequence between aminoacid 350 and aminoacid 356 as shown on figure 1

According to one embodiment of the invention, polynucleotides are administered into a patient to achieve controlled expression of the Y353 ezrin mutant or fragments or derivatives thereof.

Said polynucleotides are DNA or RNA sequences encoding the Y353 ezrin mutant or encoding fragments or derivatives thereof, operatively linked to the genetic elements necessary for their expression by a target cell, such as promoters and the like.

Said polynucleotides can be administered in a "naked" form, i.e. free of any delivery vehicle that can act to facilitate entry into the cell.

The DNA or RNA encoding ezrin mutated on tyrosine 353, or encoding fragments or derivatives thereof, may be inserted into an expression vector, in which it is operatively linked to components which allow its expression to be regulated, in particular such as transcription promoters and/or terminators.

Such an expression vector may be in particular a plasmid, a phage or any type of recombinant virus.

Among the prokaryotic transformation vectors which are well known to those skilled in the art, mention may be made of the ZAP Lambda phage vector and the pBluescript plasmid (Stratagene). Other vectors which are suitable for the transformation of *E. coli* cells include pET expression vectors (Novagen) for example, pET11a, which contains the T7 promoter, the T7 terminator, the *E. coli* inducible Lac operon and the Lac repressor gene ; and pET 12a-c, which contains the T7 promoter, the T7 terminator and the *E. coli* omPT secretion signal.

The vectors which are particularly preferred for the transfection of mammalian cells are vectors containing the cytomegalovirus (CMV) promoters such as pcDNA1 (Invitrogen), vectors containing the MMTV promoter such as pMAMNeo (Clontech) and pMSG (catalogue n° 27-4506-01 from Pharmacia) and vectors containing the SV40 promoter such as pSV β (Clontech).

In the present invention, a promoter refers to a DNA segment which controls the transcription of DNA to which it is operatively linked. The promoter region includes specific sequences which are sufficient for recognition of the RNA polymerases, for binding and the initiation of transcription. In addition, the promoter region includes sequences which modulates this recognition, and the initiation of the binding and of the transcription of the RNA polymerase activity. As examples of promoters considered for use in the present invention, mention may be made of the SV40 promoter, the cytomegalovirus promoter, the mouse mammary tumour virus promoter (induced by steroids) and the Maloney murine leukemia virus promoter.

The polynucleotides encoding Y353 ezrin mutant or fragments or derivatives thereof can be also administered along with any material that promotes transfection, such as liposomal formulations, charged lipids such as Lipofectin™ or precipitating agents such as CaPO₄.

The pharmaceutical compositions of the invention may be administered to any animal which may experience the beneficial effects of the

compositions of the invention. Foremost among such animals are humans, although the invention is not intended to be so limited.

DNA or RNA sequences encoding the Y353 ezrin mutant or encoding functional fragments or derivatives thereof can be administered to the
5 patient by any method that delivers injectable materials to cells of the patient, such as by injection into the interstitial space of tissues such as muscles or skin, introduction into the circulation or into body cavities or by inhalation or insufflation. A naked polynucleotide is injected or otherwise delivered to the animal with a pharmaceutically acceptable liquid carrier. For all applications,
10 the liquid carrier is aqueous or partly aqueous, comprising sterile, pyrogen-free water. The pH of the preparation is suitably adjusted and buffered.

The pharmaceutical compositions of the present invention (containing an effective amount of ezrin mutated on tyrosine 353, or a
15 functional fragment or derivative thereof or containing an effective amount of DNA or RNA encoding ezrin mutated on tyrosine 353, or encoding functional fragments or derivatives thereof), may be administered by any means that achieve the intended purpose. For example, administration may be by parenteral, subcutaneous, intravenous, intradermal, intramuscular,
20 intraperitoneal, or transdermal routes. Alternatively, or concurrently, administration may be by the oral route. The peptides and pharmaceutical compositions can be administered parenterally by bolus injection or by gradual perfusion over time.

Ezrin mutated on tyrosine 353, or a functional fragment or
25 derivative thereof or DNA or RNA encoding ezrin mutated on tyrosine 353, or encoding functional fragments or derivatives thereof must be targeted toward tumor cells. For that purpose, the pharmaceutical compositions of the invention can be administered by direct injection to tumor aggregates.

Specific targeting to tumor cells may also be achieved by coupling
30 Y353 ezrin mutant peptide with an antibody which specifically recognizes tumor cells. Y353 ezrin mutant peptide may also be delivered to tumor cells by liposomes carrying antibodies specific for tumor cells.

The dosage administered will be dependent upon the age, sex, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

5 The dose ranges for the administration of the composition of the present invention are those large enough to produce the desired effect. The doses should not be so large as to cause adverse side effects.

Preferred doses of the Y353 ezrin mutant peptide for humans range between about 10^{-9} - 10^{-3} moles for one intratumoral injection. Injections are performed as necessary, the rate and amount being determined by the
10 practioner.

In addition to the Y353 ezrin mutant and its derivatives which themselves are pharmacologically active, pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients
15 and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically.

To enhance delivery or bioactivity, the peptides (Y353 ezrin mutant and its derivatives) can be incorporated into liposomes using methods and compounds known in the art.
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A further subject of the present invention is a method for the prevention and/or treatment of tumors, more particularly for the prevention and/or treatment of metastasis, comprising the administration to a patient in need of such treatment of an effective amount of a pharmaceutical composition
25 according to the present invention.

All kinds of tumors are comprised. Tumors which are very likely to lead to metastasis are preferably aimed at. Examples of such tumors include epithelial cell tumors such as melanoma, and carcinoma.

30 The following examples and figures are intended to be illustrative but not to limit the invention.

LEGENDS TO THE FIGURES :

- Figure 1 represents the aminoacid sequence of human ezrin before the maturation by deletion of the first aminoacid Met.

5 - Figure 2 shows the establishment of LLC-PK1 cell lines overexpressing wild type ezrin (E1, E2) or ezrin mutant tyr 353 to Phe (F1, F2).

The level of ezrin in cells overexpressing wild type ezrin (E) and ezrin mutant (F) was compared to the level of endogenous ezrin in cells transfected with the vector alone (P).

10 Figure 2a shows an immunodetection performed with anti-tag antibody. Tagged ezrin is only detected in transfected cells.

Figure 2b shows an immunodetection performed with the anti ezrin antibody.

15 Figure 2c shows an immunolocalization of ezrin in LLC-PK1 cells overproducing wild-type ezrin (top panel) or ezrin mutant (bottom panel).

- Figure 3 shows three cell lines (E : cells overexpressing wild type ezrin ; F : cells overexpressing ezrin mutant ; P : cells transfected with the vector alone), grown in a collagen matrix in presence of HGF. F cells undergo apoptosis.

20 - Figure 4 shows three cell lines (E : cells overexpressing wild type ezrin ; F : cells overexpressing ezrin mutant ; P : cells transfected with the vector alone), grown in Matrigel® gels. Cells do not form tubules. F cells do not undergo apoptosis.

25 - Figure 5 shows three cell lines (E : cells overexpressing wild type ezrin ; F : cells overexpressing ezrin mutant ; P : cells transfected with the vector alone), grown in poly HEMA coated dishes. F cells undergo apoptosis. Piknotic nuclei are observed in cell aggregates.

- Figure 6 shows an estimation of the percentage of apoptotic cells grown on poly HEMA compared to cells grown on plastic dishes.

30 - Figure 7 shows DNA ladder showing apoptosis in F cells and also shows cells overexpressing Bcl-2 (B) and F353 cells transfected with the

vector alone (H), grown in a collagen matrix. Bcl-2 can preserve the cells from apoptosis.

- Figure 8 is a graph showing LLC-PK1 cell survival in function of the concentration of the PI3-K inhibitor, LY 294002.

5 - Figure 9 shows an SDS-PAGE electrophoresis with the peptide : biotin-RQIKIWFQNRRMKWKKLRLQDYEEKTK, or with the phosphorylated peptide : biotin-RQIKIWFQNRRMKWKKLRLQD(pY)EEKTK. Lane 0 is blank. The PI3-K-p85 subunit specifically interacts with the phosphorylated peptide.

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EXAMPLES :

EXAMPLE 1 : Construction of ezrin mutated on tyrosine 353

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Materials :

LLC-PK1 (CCL 101; ATCC) cells were grown in DMEM growth medium (GIBCO BRL) supplemented with 10% fetal calf serum (FCS) and maintained at 37°C in 10% CO₂.

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Rabbit polyclonal anti-ezrin antibody was raised against the entire ezrin produced in bacteria and was previously described (Algrain *et al.*, 1993).

DNA constructs and transfection :

For generating the plasmid producing ezrin mutated on tyrosine 25 353 the following constructs were made. To make the F353 mutant (wherein Tyr353 is replaced by Phe353), the two oligonucleotides :

5' CGGAATTCCGGCTGCAGGACTTTGAGGAG 3' and

5' CGCGGATCCATTGTGGGTCC TCTTA 3' flanked with EcoRI and BamHI restriction sites respectively, were used to amplify the fragment 30 (nucleotides 1125 to 1730) using the Ampli Taq polymerase (Perkin-Elmer Corp., Norwalk, CT). This fragment was then subcloned into the Bluescript plasmid and checked by double-strand DNA sequencing using the T7

sequencing kit (Pharmacia FineChemicals, Piscataway, NJ). The fragment PstI-PstI corresponding to the sequence 1131 to 1197 and containing the mutated codon was inserted into the fragment Aval-Aval (nucleotides 1002-1698) in the plasmid psp64. The fragment Aval-Aval of the full length ezrin cDNA in the plasmid psp64 was then replaced by the fragment Aval-Aval containing the mutated codon. This mutant ezrin cDNA was then cloned in the expression vector pCB6 through the HindIII and XbaI restriction sites.

Exponentially growing LLC-PK1 cells were seeded 24 h before DNA transfer on 10-cm tissue culture dishes. DNA transfer was performed following the procedure of Chen and Okayama, (1987) and cells transfected were selected by growing in media containing 0.7mg/ml Généticine (G-418), [Gibco BRL (Life Technologies) ref 11811-049] for 2-3 weeks.

For each transfection, three/four clones overproducing the transfected protein (as detected by immunoblot and immunofluorescence analysis) were selected for further study.

To generate the F353/ bcl-2 cell lines, the F353 LLC-PK1 cells (clone 7) were transfected with 20 µg of plasmid RSV-tk-hygromycin or plasmid RSV-tk- hygromycin- hbcl-2 by electroporation (240V and 950-µF) with the Gene Pulser II System (Bio-Rad, Hercules, Ca.). Transfected cells were selected with 0.2mg/ml of hygromycin B in presence of 0.7 mg/ml G418. After 3 weeks of selection, individual colonies were isolated and expanded into cell lines.

EXAMPLE 2 : Establishment of LLC-PK1 cell lines overexpressing wild type ezrin or Y353 ezrin

Materials

P5D4 mAb raised against the 11-amino acid carboxy terminus of the vesicular stomatitis virus glycoprotein G (VSV-G peptide), was previously described (Kreis, 1986).

Results

Figure 2 shows the establishment of LLC-PK1 cell lines overexpressing wild type ezrin (E1, E2) or ezrin mutant tyr 353 to Phe (F1, F2).

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The level of ezrin in cells overexpressing wild type ezrin (E) and ezrin mutant (F) was compared to the level of endogenous ezrin in cells transfected with the vector alone (P). Wild type and mutant ezrin were tagged with the VSV-G peptide at their carboxy-terminus. The immunodetection performed with the anti ezrin antibody (Fig 2B) shows that the level of transfected ezrin is 5-10 fold higher than the endogenous ezrin (lane P). The localization of ezrin mutant is similar to that of wild type ezrin in LLC-PK1 cells (Fig 2C). There is not a striking morphological change when the cell lines are grown on plastic dishes.

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EXAMPLE 3 : Biological assays

Materials and methods

For the tubulogenesis assay in three-dimensional collagen gels, the trypsinized cells were suspended at a final concentration of 1×10^5 cells/ml in gelling solution, prepared as follows 1 part of DMEM 10x (GIBCO BRL), 1 part of NaHCO_3 (37g/l), 1 part of FCS were mixed with 3.5 parts of a suspension of 3×10^5 cells/ml and 3.5 parts of type I collagen at 5mg/ml (Collaborative Biomedical Products) at room temperature. 100 μ l of this mixture was seeded, in a microtiter plate, onto 100 μ l of a first layer of collagen without cell suspension. After 5 min at 37°C. the gels were covered with cell culture medium +/- 100 U/ml HGF (dia-filtered, human fibroblast MRC5-conditioned medium (Naldini et al., 1995)). Photographs were taken with a light microscope (Leica) equipped with Nomarski interference optics. To assess apoptosis, the cells in the collagen preparation were fixed with methanol for 5 min and stained with HOECHST 33258 (10 μ g/ml). The preparations were examined with a

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Leica microscope equipped with a UV-absorbing filter. Condensed and fragmented nuclei were easily distinguishable from intact nuclei and percentage were calculated by counting.

Growth of LLC-PK1 cell lines was tested in a growth factor reduced Matrigel®, a soluble basal membrane extract which do not contain collagen type I (Becton Dickinson Labware, Bedford, MA.). 1×10^5 cells (1 v) were mixed with Matrigel® (5 v) and gelation occurred at 37°C.

Results :

1. The survival of ezrin mutant is impaired in a collagen matrix (figure 3).

When the three cell lines P, E, F were grown in a collagen matrix in presence of HGF striking differences between the three cell lines were observed. On the left panels, photographs were taken with a light microscope (Leica) equipped with Normarski interference optics. On the right panels, nuclei are labelled with the intercalating dye, Hoechst 33258. P and E cells developed tubules with more ramifications when cells overexpressed wild type ezrin (E). However no tubulogenesis is observed with F cells. The few cells observed present fragmented nuclei typical of apoptosis (F). This suggested that ezrin mutant impairs the ability of cells to survive in a collagen matrix.

2. Ezrin mutant survive in Matrigel® gels (figure 4).

The ability of the three cell lines to grow in a Matrigel® gel was tested. All three cell lines were able to form cysts in the matrix and the F cell line did not undergo apoptosis as shown by analysis of the nuclei with the Hoechst dye (left panels). This indicated that ezrin is in the survival pathway elicited by collagen type I matrix and not by Matrigel®.

3. Cell survival mediated by ezrin is adhesion dependent (figures 5-6).

Growth of epithelial cells is anchorage dependent. When the three

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cell lines were grown on an anti-adhesive substrate, the poly HEMA coated dishes, P and E cells escape apoptosis through aggregation and formation of cysts (figure 5). In contrast, a significant proportion of F cells underwent apoptosis within aggregates. Apoptosis was estimated by two approaches. First DNA ladder (figure 6) shows that a higher level of DNA fragmentation occurred in F cells compared to P or E cells. Flow cytometric analysis allowed to estimate the percentage of apoptotic cells grown on poly HEMA compared to cells grown on plastic dishes. While the percentage of apoptotic cells is the same for all three clones grown on plastic dishes, the percentage of apoptotic cells is 2-3 times higher in F cells.

4. Bcl-2 overexpression rescues F353 ezrin mutants from apoptosis (figure 7).

In order to confirm that apoptosis is due to a defect in signal transduction mediated by ezrin, cell lines overexpressing Bcl-2 from F353 cells have been established. The double transfected cell lines were tested for their ability to survive in a collagen matrix. Fig 7 shows that cells overexpressing Bcl-2 survive in a collagen matrix (Fig 7B) while F353 cells transfected with the vector alone do not (Fig 7H). In addition, Bcl-2 transfected cells were able to form tubules, indicating that they did not lose their ability to interact with the collagen matrix.

Altogether these data indicate that ezrin is involved in a specific pathway mediating cell survival.

Furthermore, since PI3-K (PI3-kinase) has been shown to play a role in adhesion-dependent cell survival, it could be involved in this pathway.

EXAMPLE 4 : Treatment of LLC-PK1 cells with the PI 3-kinase inhibitor, LY294002

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Materials and methods :

The effect of LY294002 (Sigma ref. L9908) was assessed on

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LLC-PK1 cells grown in type I collagen or Matrigel® matrices. 10^5 cells/ml were embedded in the matrices and cultured, as previously described, in presence of 100 U/ml HGF. Cells were treated with LY294002 at increasing concentrations or with the vehicle DMSO. After 24 h, cultures were permeabilized with several changes of methanol 100% for 30 min at -20°C, and nuclei were stained with Hoechst 33258 (10 mg/ml in phosphate buffer saline). Condensed and intact nuclei were scored under microscope (Leica).

Results :

Figure 8 shows that LLC-PK1 cell survival is impaired by the PI3-K inhibitor, LY294002.

LY294002 is a specific inhibitor of PI3-K. When cells were grown in a collagen matrix in presence of various concentration of inhibitor, we observed a strong inhibition of cell survival at a concentration of LY294002 as low as 20 μ M. No apoptosis is observed at the same concentration when the cells are grown in a Matrigel® matrix. This indicates that the cell survival signal elicited by the collagen matrix is PI3-K and ezrin dependent.

EXAMPLE 5 : p85 interaction with phosphorylated peptides

Materials and methods :

The authors of the present invention have designed peptides that contain a biotin fused to the Antennapedia internalization sequence (Derossi et al., 1994) in tandem with an eleven amino-acid peptide corresponding to the ezrin amino-acids 348-358. In one peptide, the tyrosine 353 was phosphorylated. The peptide sequence is : biotin-RQIKIWFQNRRMKWKKLRLQDY(p)EEKTK.

25 μ l of Streptavidin Ultralink beads (Pierce, Rockford, IL, USA) were pre-incubated with 300 μ g of biotinylated peptides for 1 h at 4°C in buffer A (50 mM Hepes pH 7.4, 2 mM EDTA, 1% Triton X-100, 100 mM NaCl, 50 mM ammonium molybdate, 1 mM $ZnCl_2$). 7.10^6 LLC-PK1 cells grown on

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plastic dishes, were lysed with cold A buffer supplemented with a cocktail of protease inhibitors. Extracts were clarified by centrifugation, 10 min at 12000 g at 4°C. The beads were incubated with the extracts for 1 h, washed 3 times with buffer A, and re-suspended in SDS loading buffer. Samples were boiled
5 and submitted to electrophoresis on SDS-PAGE.

Results :

Figure 9 shows that Ezrin phosphorylated peptide (aa 348-358) interacts with the PI3-K p85 subunit.

10 The authors of the present invention made the hypothesis that interaction of the ezrin mutant with PI3-K was altered. To test this hypothesis, an affinity column was performed with the ezrin peptides phosphorylated or not. As shown in Fig 9 an interaction of PI3-K p85 subunit is only observed with the phosphorylated peptide.

15

Altogether the above results indicate that :

- Ezrin is involved in adhesion -dependent cell survival of epithelial cells.

- Ezrin controls cell survival by activating the PI3-K whose
20 downstream target, in this pathway, is the serine/threonine kinase Akt.

- This control is mediated by the interaction of the ezrin phosphorylated tyrosine residue 353 with the p85 subunit of the PI3-K.

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25

CLAIMS

5 1. A pharmaceutical composition containing an effective amount of ezrin mutated on tyrosine 353, or a functional fragment or derivative thereof, in association with a pharmaceutically acceptable carrier.

 2. A pharmaceutical composition containing an effective
10 amount of DNA or RNA encoding ezrin mutated on tyrosine 353, or encoding functional fragments or derivatives thereof.

 3. A pharmaceutical composition according to claim 2 wherein said DNA or RNA is in a naked form.

15

 4. A pharmaceutical composition according to any of claims 1 to 3 useful for the prevention and/or treatment of tumors.

 5. A pharmaceutical composition according to any of claims 1
20 to 4 useful for the prevention and/or treatment of metastasis.

 6. A method for the prevention and/or treatment of tumors, more particularly for the prevention and/or treatment of metastasis, comprising the administration to a patient in need of such treatment of an effective amount
25 of a pharmaceutical composition according to any of claims 1 to 5.

1
M P K P I N V R V T T M D A E L E F A I 19
Q P N T T G K Q L F D Q V V K T I G L R 39
E V W Y F G L H Y V D N K G F P T W L K 59
L D K K V S A Q E V R K E N P L Q F K F 79
R A K F Y P E D V A E E L I Q D I T Q K 99
L F F L Q V K E G I L S D E I Y C P P E 119
T A V L L G S Y A V Q A K F G D Y N K E 139
V H K S G Y L S S E R L I P Q R V M D Q 159
H K L T R D Q W E D R I Q V W H A E H R 179
G M L K D N A M L E Y L K I A Q D L E M 199
Y G I N Y F E I K N K K G T D L W L G V 219
D A L G L N I Y E K D D K L T P K I G F 239
P W S E I R N I S F N D K K F V I K P I 259
D K K A P D F V F Y A P R L R I N K R I 279
L Q L C M G N H E L Y M R R R K P D T I 299
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Q Q L E T E K K R R E T V E R E K E Q M 339
M P E K E E L M L R L Q D Y E E K T K K 359
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A R D E N K R T H N D I I H N E N M R Q 559
G R D K Y K T L R Q I R Q G N T K Q R I 579
D E F E A L *

FIG. 1

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Characterization of stable LLC-PK1 transfectants

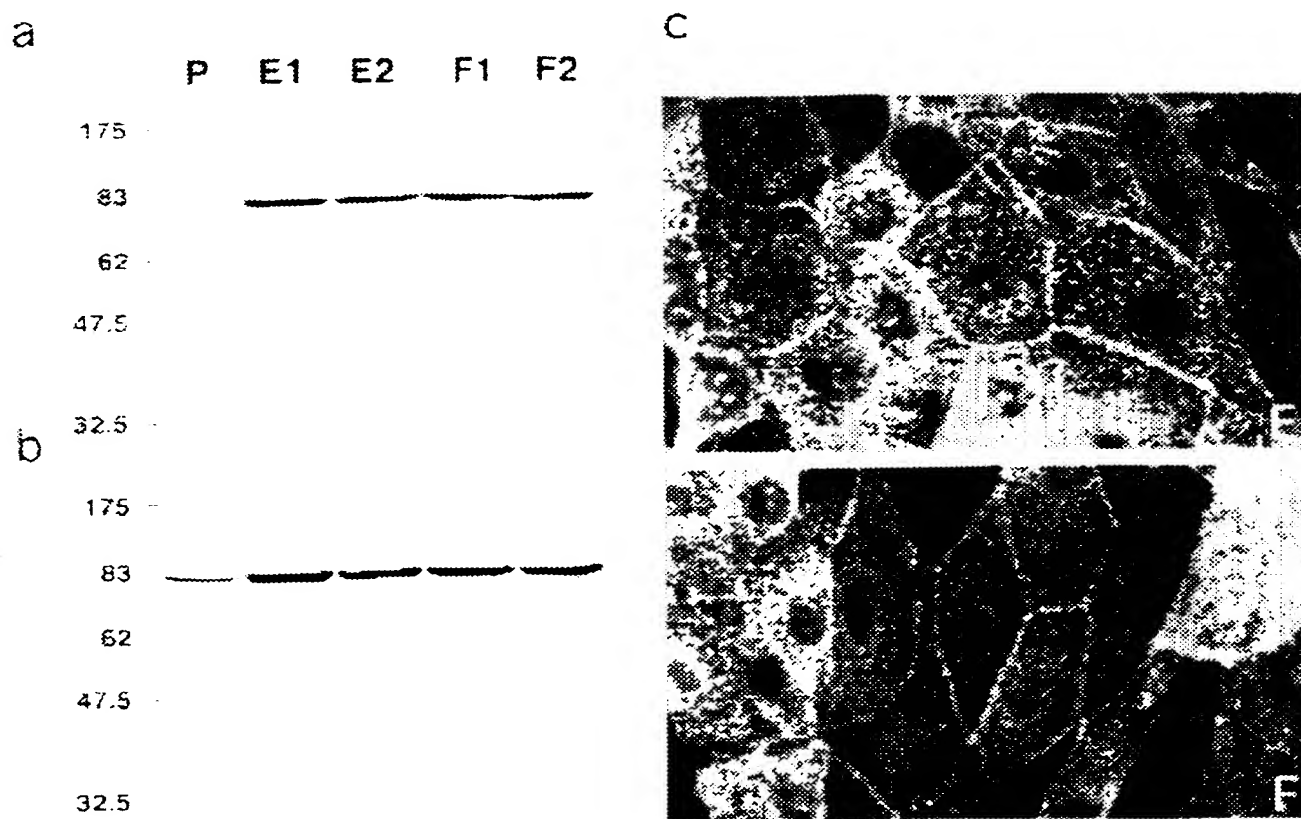


Fig. 2

F353 ezrin mutants undergo
apoptosis in collagen gels

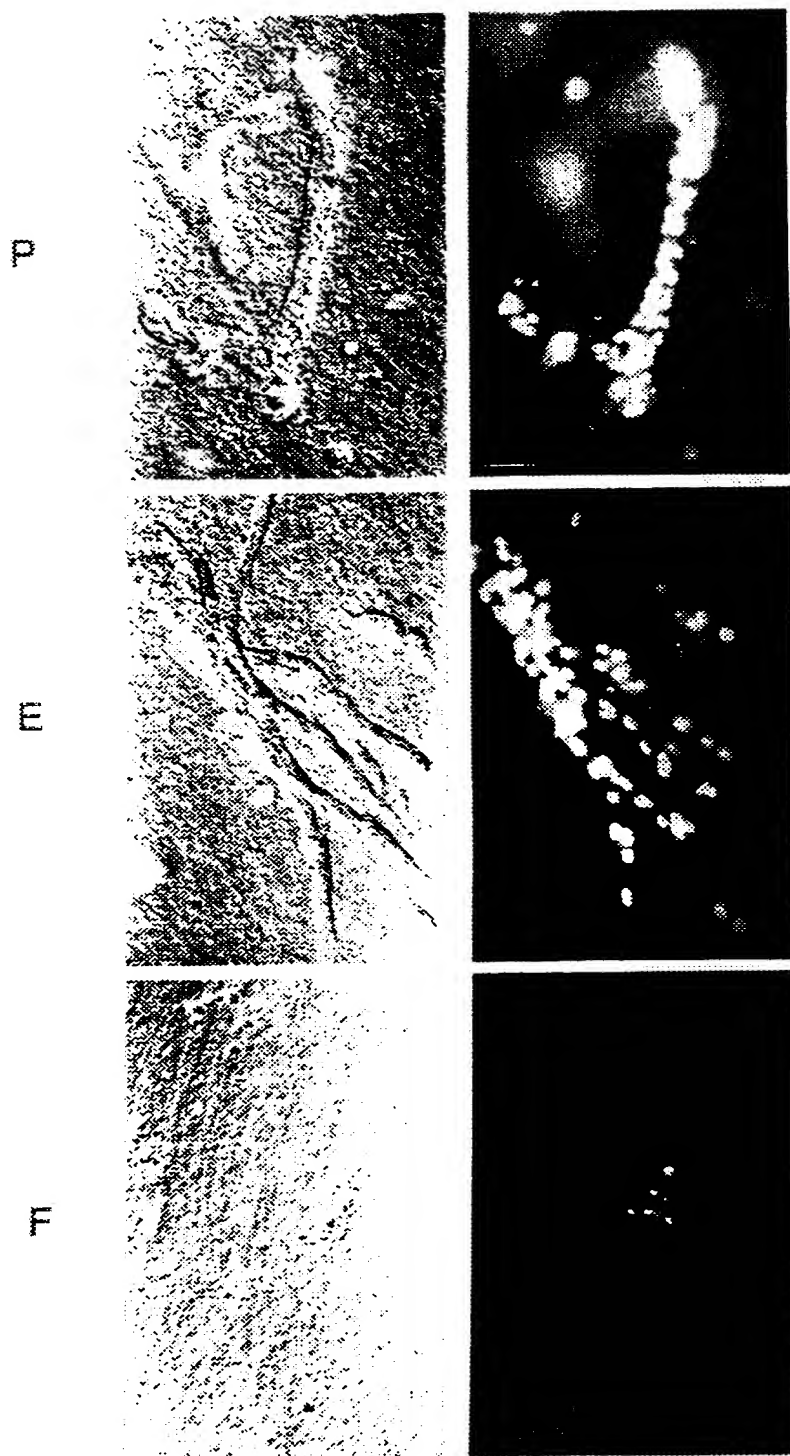


Fig. 3

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F353 ezrin mutants survive in Matrigel gels

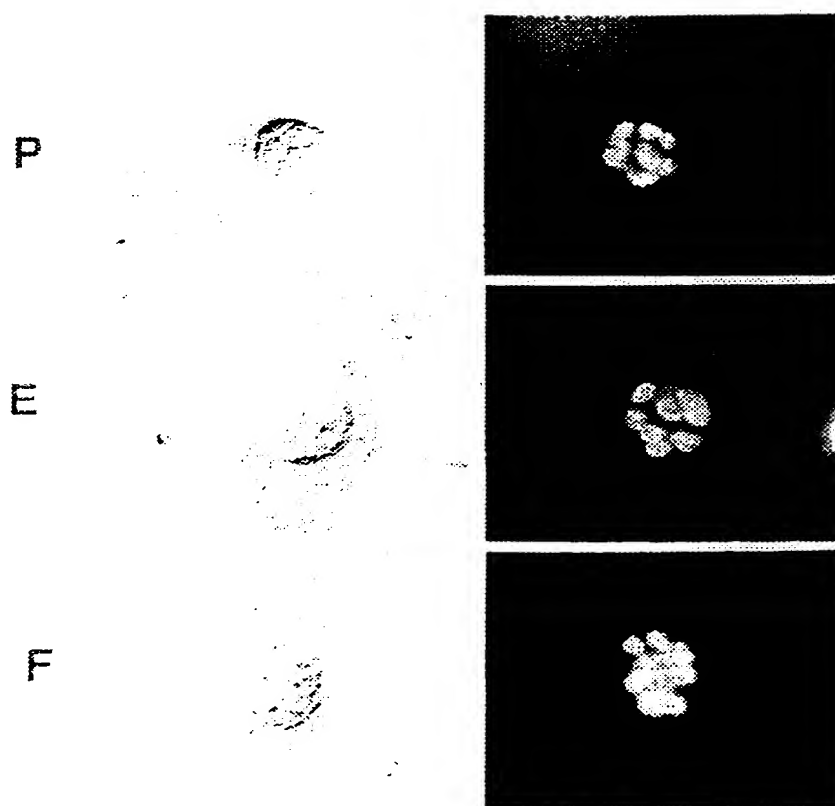


Fig. 4

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F353 ezrin mutants undergo
apoptosis in cellular aggregates

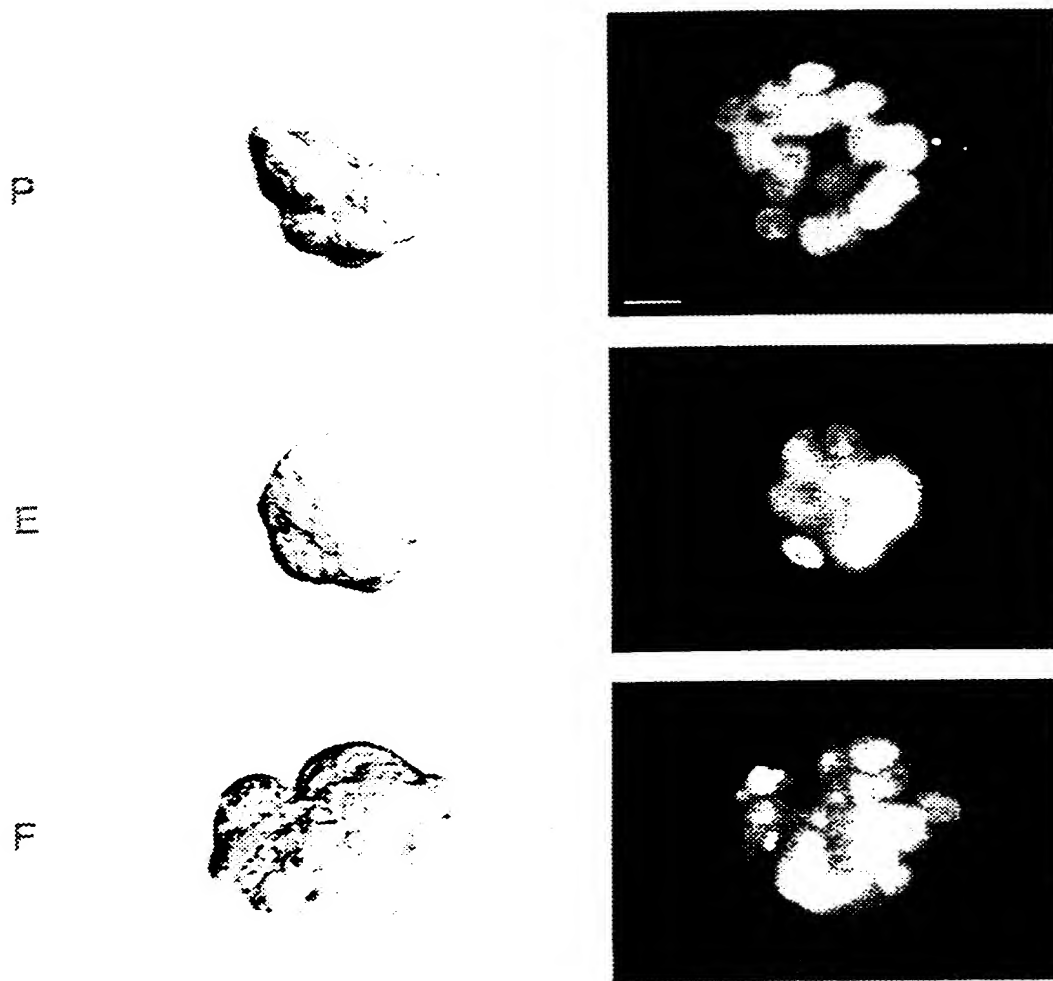
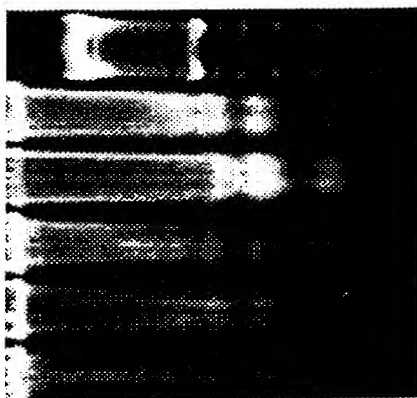
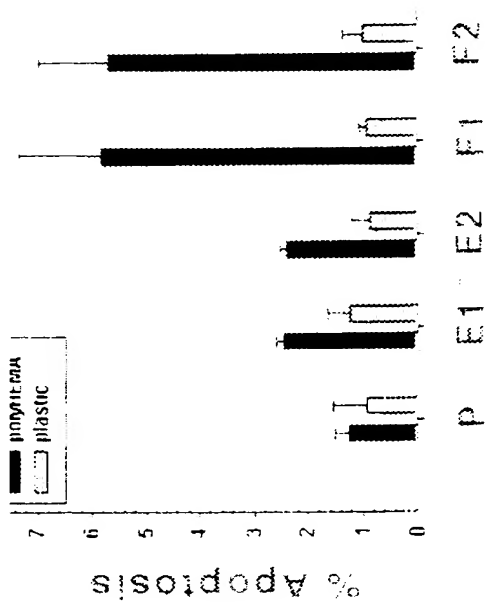


Fig 5

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Fig. 6



Bcl-2 expression rescues F353 ezrin mutants from apoptosis and allows tubulogenesis

A

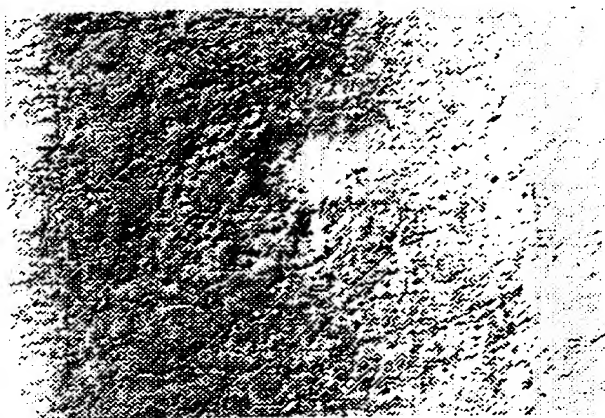
H1 H2 B1 B2

Tag

Bcl-2

B

H



B

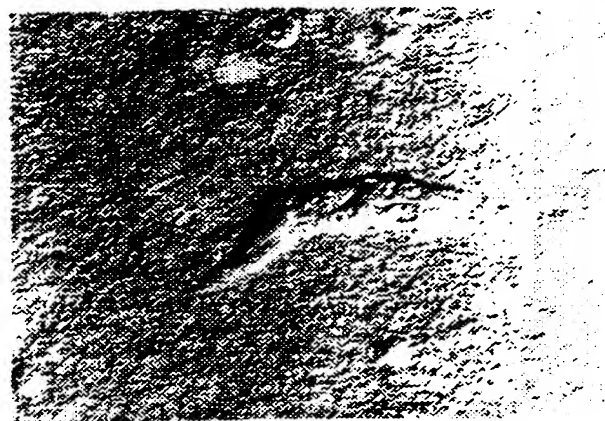
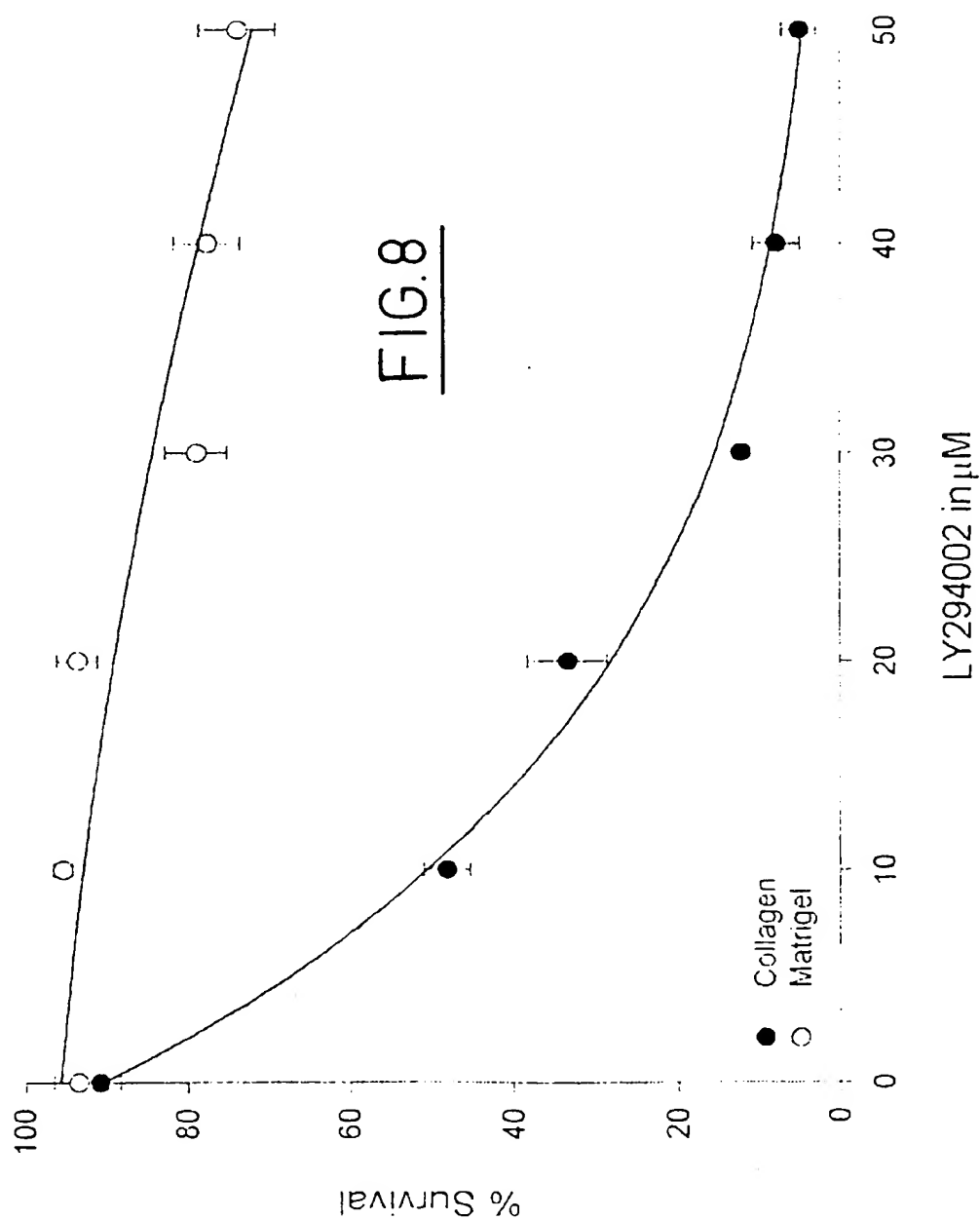


Fig.7

**LY294002 strongly inhibits LLC-PK1 survival
in collagen but not in Matrigel matrices**



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Ezrin 348-358 interacts with PI3K p85
in a phosphorylation-dependent manner

Biotin		P
1		
<u>RQIKIWFFQNRRMKWKKLRLQDYEEKTK</u>		
Antennapedia		Ezrin 348-358
cell-permeable peptide		

0	Y	pY
	—	p85

Fig. 9

SEQUENCE LISTING

<110> Institut Curie
CNRS

<120> A pharmaceutical composition containing ezrin mutated
on tyrosine 353

<130> BET 99/0268

<140>

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<150> US/040,725

<151> 1998-03-18

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35 40 45

Tyr Val Asp Asn Lys Gly Phe Pro Thr Trp Leu Lys Leu Asp Lys Lys
50 55 60

Val Ser Ala Gln Glu Val Arg Lys Glu Asn Pro Leu Gln Phe Lys Phe
65 70 75 80

Arg Ala Lys Phe Tyr Pro Glu Asp Val Ala Glu Glu Leu Ile Gln Asp
85 90 95

Ile Thr Gln Lys Leu Phe Phe Leu Gln Val Lys Glu Gly Ile Leu Ser
100 105 110

Asp Glu Ile Tyr Cys Pro Pro Glu Thr Ala Val Leu Leu Gly Ser Tyr

115	120	125
Ala Val Gln Ala Lys Phe Gly Asp Tyr Asn Lys Glu Val His Lys Ser		
130	135	140
Gly Tyr Leu Ser Ser Glu Arg Leu Ile Pro Gln Arg Val Met Asp Gln		
145	150	155
His Lys Leu Thr Arg Asp Gln Trp Glu Asp Arg Ile Gln Val Trp His		
	165	170
Ala Glu His Arg Gly Met Leu Lys Asp Asn Ala Met Leu Glu Tyr Leu		
	180	185
Lys Ile Ala Gln Asp Leu Glu Met Tyr Gly Ile Asn Tyr Phe Glu Ile		
	195	200
Lys Asn Lys Lys Gly Thr Asp Leu Trp Leu Gly Val Asp Ala Leu Gly		
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Leu Asn Ile Tyr Glu Lys Asp Asp Lys Leu Thr Pro Lys Ile Gly Phe		
	225	230
Pro Trp Ser Glu Ile Arg Asn Ile Ser Phe Asn Asp Lys Lys Phe Val		
	245	250
Ile Lys Pro Ile Asp Lys Lys Ala Pro Asp Phe Val Phe Tyr Ala Pro		
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Arg Leu Arg Ile Asn Lys Arg Ile Leu Gln Leu Cys Met Gly Asn His		
	275	280
Glu Leu Tyr Met Arg Arg Arg Lys Pro Asp Thr Ile Glu Val Gln Gln		
	290	295
Met Lys Ala Gln Ala Arg Glu Glu Lys His Gln Lys Gln Leu Glu Arg		
	305	310
Gln Gln Leu Glu Thr Glu Lys Lys Arg Arg Glu Thr Val Glu Arg Glu		
	325	330
Lys Glu Gln Met Met Arg Glu Lys Glu Glu Leu Met Leu Arg Leu Gln		
	340	345
Asp Tyr Glu Glu Lys Thr Lys Lys Ala Glu Arg Glu Leu Ser Glu Gln		
	355	360
Ile Gln Arg Ala Leu Gln Leu Glu Glu Glu Arg Lys Arg Ala Gln Glu		

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Glu Glu Leu Glu Arg Gln Ala Val Asp Gln Ile Lys Ser Gln Glu Gln		
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Leu Ala Ala Glu Leu Ala Glu Tyr Thr Ala Lys Ile Ala Leu Leu Glu		
420	425	430
Glu Ala Arg Arg Arg Lys Glu Asp Glu Val Glu Glu Trp Gln His Arg		
435	440	445
Ala Lys Glu Ala Gln Asp Asp Leu Val Lys Thr Lys Glu Glu Leu His		
450	455	460
Leu Val Met Thr Ala Pro Pro Pro Pro Pro Pro Pro Val Tyr Glu Pro		
465	470	475 480
Val Ser Tyr His Val Gln Glu Ser Leu Gln Asp Glu Gly Ala Glu Pro		
485	490	495
Thr Gly Tyr Ser Ala Glu Leu Ser Ser Glu Gly Ile Arg Asp Asp Arg		
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Asn Glu Glu Lys Arg Ile Thr Glu Ala Glu Lys Asn Glu Arg Val Gln		
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Arg Gln Leu Val Thr Leu Ser Ser Glu Leu Ser Gln Ala Arg Asp Glu		
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Asn Lys Arg Thr His Asn Asp Ile Ile His Asn Glu Asn Met Arg Gln		
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20 25

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<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: primer
(example 1)

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cggaattccg gctgcaggac ttgaggag

29

<210> 4

<211> 25

<212> DNA

<213> Artificial Sequence

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<223> Description of Artificial Sequence: primer
(example 1)

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cgcggatcca ttgtgggtcc tctta

25





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 38/17 // C12N 15/12, C07K 14/47	A3	(11) International Publication Number: WO 99/47150 (43) International Publication Date: 23 September 1999 (23.09.99)
<p>(21) International Application Number: PCT/EP99/02054</p> <p>(22) International Filing Date: 18 March 1999 (18.03.99)</p> <p>(30) Priority Data: 09/040,725 18 March 1998 (18.03.98) US</p> <p>(71) Applicants (for all designated States except US): INSTITUT CURIE [FR/FR]; 26, rue d'Ulm, F-75248 Paris Cedex 05 (FR). CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE (C.N.R.S.) [FR/FR]; 3, rue Michel Ange, F-75016 Paris (FR).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): ARPIN, Monique [FR/FR]; 9, rue Roli, F-75014 Paris (FR). CREPALDI, Tiziana [IT/IT]; Piazza Chiaves, 4, I-10153 Torino (IT). GAUTREAU, Alexis [FR/FR]; 5, rue du Paradis, F-75010 Paris (FR). LOUVARD, Daniel [FR/FR]; 23, allée de Tréville, F-92330 Sceaux (FR).</p> <p>(74) Agent: MONCHENY, Michel; Cabinet Lavoix, 2, place d'Estienne d'Orves, F-75441 Paris Cedex 09 (FR).</p>	<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: 20 January 2000 (20.01.00)</p>	
<p>(54) Title: A PHARMACEUTICAL COMPOSITION CONTAINING EZRIN MUTATED ON TYROSINE 353</p> <p>(57) Abstract</p> <p>The invention relates to a pharmaceutical composition containing an effective amount of ezrin mutated on tyrosin 353, or a functional fragment or derivative thereof, in association with a pharmaceutically acceptable carrier, for use preferably in the prevention and/or treatment of tumors.</p>		

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INTERNATIONAL SEARCH REPORT

Application No

PCT/EP 99/02054

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K38/17 //C12N15/12, C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>T. CREPALDI ET AL.: "EZRIN IS AN EFFECTOR OF HEPATOCYTE GROWTH FACTOR-MEDIATED MIGRATION AND MORPHOGENESIS IN EPITHELIAL CELLS." THE JOURNAL OF CELL BIOLOGY., vol. 138, no. 2, 28 July 1997 (1997-07-28), pages 423-434, XP002122975 ROCKEFELLER UNIVERSITY PRESS., US ISSN: 0021-9525 cited in the application page 424, right-hand column, line 15 - line 19 page 429, right-hand column, line 18 - line 20 page 430, left-hand column, paragraph 1 page 432, right-hand column, line 3 - line 10</p> <p style="text-align: center;">-/-</p>	1-6

☒ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

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Date of the actual completion of the international search

17 November 1999

Date of mailing of the international search report

03/12/1999

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/02054

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>T. KONDO ET AL.: "ERM (EZRIN/RADIXIN/MOESIN)-BASED MOLECULAR MECHANISM OF MICROVILLAR BREAKDOWN AT AN EARLY STAGE OF APOPTOSIS." THE JOURNAL OF CELL BIOLOGY., vol. 139, no. 3, 3 November 1997 (1997-11-03), pages 749-758, XP002122976 ROCKEFELLER UNIVERSITY PRESS., US ISSN: 0021-9525 page 749, abstract</p>	1-6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 99/ 02054

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 6 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

